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IMPACT OF MEDIUM AND SUBSTRATE ON GROWTH OF PSEUDOMONAS FLUORESCENS BIOFILMS ON POLYURETHANE PAINT

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14. ABSTRACT
Since the 1950s, the Air Force has battled microbial contamination of its fuel systems. Within these systems, microbes are commonly found as biofilms adhered to tank coatings. Thus strategies for mitigating microbial contamination of fuel systems include deterring biofilm growth on coatings. This study investigated how Pseudomonas fluorescens biofilms grow on polyurethane coatings, and contribute to the degradation of those coatings. Specifically, we characterized how medium conditions and substrate composition contribute to growth of P. fluorescens on an antistatic polyurethane coating. We found that P. flourescens can use polyurethane as a nutrient source. In addition, we examined the impact of substrate on biofilm growth by quantitating biofilm growth on pigmented and non-pigmented paint. There was no difference in the ability of the biofilm to grow on the pigmented vs. non-pigmented paint, suggesting that the biofilm was utilizing the polyurethane and not the pigment as a carbon source.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

Since the 1950s, the Air Force has battled microbial contamination within its fuel distribution chain. Microbes in fuel cause degradation of fuel quality, clogging of fuel lines and filters, degradation of tank coatings, and corrosion of fuel tanks. Within these systems, microbes are commonly found as biofilms, communities that are adhered to abiotic and biotic surfaces. It follows that strategies for mitigating microbial contamination of fuel systems should concentrate on deterring biofilm growth. This study investigated how *Pseudomonas fluorescens* biofilms grow on polyurethane coatings, and contribute to the degradation of those coatings. Specifically, we characterized how medium conditions and substrate composition contribute to growth of *P. fluorescens* on an antistatic polyurethane coating. We found that *P. fluorescens* biofilms grew rapidly in systems where the only carbon source was the coupon itself, suggesting that *P. fluorescens* can use the coating as a nutrient source. In addition, we examined the impact of substrate on biofilm growth by quantitating biofilm growth on pigmented and non-pigmented paint. There was no difference in the ability of the biofilm to grow on the pigmented vs.non-pigmented paint, suggesting that the biofilm was utilizing the polyurethane and not the pigment as a carbon source.

1. Summary

Historically, microbial contamination of fuel systems has been a major issue for the Department of Defense, and has been the subject of several Air Force research studies since the 1950s. Microbes cause degradation of fuel quality, fuel tank coatings, and the fuel tank itself. Degradation of fuel tank coatings is attributable to the formation of microbial biofilms on the coatings. The purpose of the current study was to investigate the parameters that affect *Pseudomonas fluorescens* biofilm formation on polyurethane (PU) coatings, and to define how those parameters contribute to polyurethane biodegradation. We used a batch flow system to expose aluminum 2024 coupons coated with an AF antistatic polyurethane coating to *P. fluorescen*s. Exposures were performed for 35 days, with time points taken at 4h, 1d, 4d, 7d, 14d, 28d, and 35d. At each time interval we monitored the number of cells that were present on the coupons and characterized biofilm morphology and coating degradation by scanning electron microscopy. Using these techniques, we evaluated the impact of medium conditions and substrate composition on biofilm growth and coating degradation.

Biofilms grew well on coupons in the absence of any carbon source other than the coupon, implying that *P. fluorescens* was utilizing PU as a carbon source. Biofilm density reached its maximum by approximately 7 days and declined thereafter. The amount of carbon source present in the medium during exposure impacted the ability of *P. fluorescens* to form a biofilm on the coupon. Addition of a small amount of carbon to the medium enhanced the rate and density of biofilm growth, but did not result in increased persistence of the biofilm, suggesting that other factors may limit persistence.

The impact of the substrate on biofilm growth was also determined, by evaluating whether *P. fluorescens* was utilizing the PU or the pigment in the paint (carbon black) as a carbon source. PU coatings with and without pigment were equally supportive of biofilm growth, suggesting that *P. fluorescens* probably utilizes the PU, and not the carbon black, as a nutrient source.

Finally, there was no substantial evidence of biodegradation of the coupon coatings under any conditions tested. Although *P. fluorescens* is capable of using PU as carbon source, it is not likely a desired substrate, and its utilization as a carbon source most likely requires *P. fluorescens* to be highly metabolically active. This finding supports the need to continue to evaluate how growth conditions contribute to biodegradation.

2. Introduction

Since the 1950's, microbial contamination of aviation fuel has been a recognized problem [1, 2]. Microbial contamination of fuel compromises missions through clogging of fuel lines, injectors and filters, and degrading the fuel itself [1-3]. Microbial growth can accelerate the degradation of fuel tank coatings and cause corrosion of aluminum and stainless steel tanks and pipelines [4]. Microbial growth in fuel is unavoidable because sterility is impossible in storage and distribution systems. The issue is compounded by the fuel distribution chain, where fuel from different sources is mixed and shared, thus spreading microbes across the distribution system. Current mitigation strategies involve filtering the fuel, treating the system with biocides, and scrubbing of tanks [4]. The latter is most effective, but often requires personnel to enter the tanks, a major health hazard. Fuel filtering and biocide treatment are only short-term solutions and biocides are never effective against all microbes in the system. Thus new solutions in controlling fuel fouling are warranted.

Most of the biomass (>99%) that contaminates fuel systems exists in biofilms, communities of microbes that live on abiotic or biotic surfaces [1, 5]. Biofilms consist of microbial cells and a slime layer called the 'extracellular polymeric substance' (EPS) [5-7]. EPS contains polysaccharides, proteins, DNA, and lipids and serves to promote adhesion, facilitate breakdown of extracellular nutrients, provide a matrix for signal and genetic exchange among the cells, and protect the biofilm from physical and chemical damage. Because of their predominance in fuel systems, prevention of biofilm growth or disruption of existing biofilms are major components of mitigation strategies for contamination of fuel by microbes.

Although a biofilm may be comprised of one strain of microbe, environmental biofilms are almost always consortia of many types of microbes [7]. The metabolic, and therefore degradative, potential of the consortium is determined by the interplay between consortium and the microenviroment it inhabits [4]. These microenvironments are determined by parameters such as oxygen and nutrient levels, pH, and redox potential. Colonization occurs successively; first colonizers of the tank lining change the microenvironment near the biofilm, which facilitates the growth of other strains [4]. For example, early colonizers may degrade the coating of the fuel tank, revealing the metal underneath, which then facilitates colonization by microbes that contribute to corrosion of the metal tank. Little is known about the succession of microbes within fuel systems and it is not known if disruption of successive colonization could mitigate the problems or timescale of fuel contamination.

One potential early colonizer of fuel systems is Pseudomonad bacteria. Pseudomonads are facultative aerobes that can utilize a large variety of inorganic and organic compounds as nutrient sources [8]. They have been shown to be common constituents of aviation fuel microbiota in numerous studies [1, 2, 9-12] and some environmental isolates directly use JP-8 as a carbon source [9]. In addition, Pseudomonads can use polyurethane (PU) as a nutrient source [13, 14] and in the 1960s were reported to penetrate the PU coatings used in aircraft fuel tanks [15]. Kay and colleagues examined the degradation potential of Pseudomonads as a function of the medium present when the microbes were exposed to the coupons [16]. They found that richer medium supported growth of planktonic bacteria and facilitated degradation of the coupons, but did not quantify biofilm growth on the PU itself. Conversely, Gu et al. focused on growth of the biofilm on PU coatings, and its degradation of the coatings, but did not examine how the medium affected biofilm growth or coupon degradation [14].

In this study, we have begun to examine the combined effects of medium conditions and substrate composition to determine which factors best support the growth and persistence of *Pseudomonas fluorescens* biofilms. Factors that enhance biofilm formation and persistence will undoubtedly also lead to enhanced substrate degradation.

3. Methods, Assumptions, and Procedures

3.1 Coupons

Coupons utilized for all experiments consisted of 1X 2 cm aluminum 2024 coupons coated with CAAPCo. AS-P108 or neat resin. Coupons were sanded prior to painting to increase adhesion, but no primer was used. Both sides and all edges were coated to ensure that no metal was exposed.

3.2 Microbial exposures

Coupons were exposed in a closed-loop batch flow system consisting of a medium reservoir and daisy-chained capsules (Fig. 1). The system was run at room temperature with 0.5X M9 salts (Amresco, Inc.) or M9 salts with 10 mM pyruvate. Periodically the system was supplemented with water to accommodate a decline in buffer volume due to evaporation.

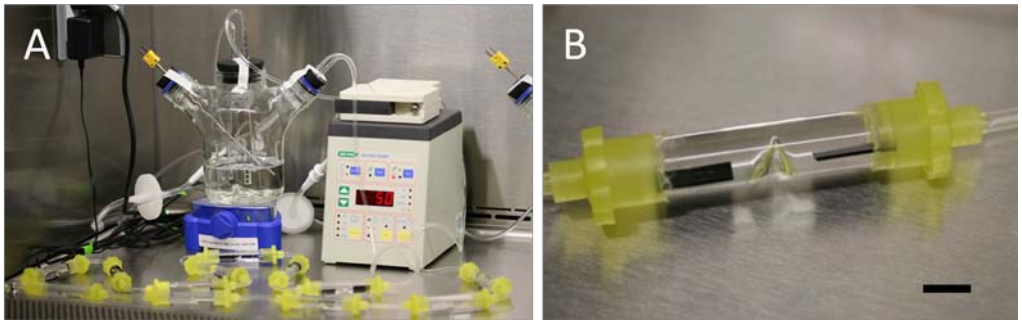

Figure 1. Closed Loop Batch Flow System for Coupon Exposure. A, complete system. B, capsule containing coupons. Scale bar, 1 cm.

The capsule system allowed removal of select coupons per time point without disturbing other capsules/coupons. It also facilitated the maintenance of axenic cultures, as confirmed by cell plating and SEM microscopy. The capsules consisted of 15mm ± 0.25mm OD glass tubing capped at each end with chromatography column caps (Bio-Rad, Inc.). Each capsule contained 2 coupons (Fig. 1B). Capsules were linked by Tygon tubing.

The main reservoir of the flow system consisted of a side arm flask containing 500 ml of medium that was aerated via sterile air generated from a standard aquarium air pump. The medium was also constantly stirred via a stir bar. Medium was pumped at 0.8 ml/min through the flow system using a peristaltic pump. To begin an exposure, system components, including coupons, were sterilized and sterile medium was flowed through the system for 24h prior to inoculation. This pre-exposure test ensured the system had not been inadvertently contaminated. To inoculate the system, a single *Pseudomonas fluorescens* (ATCC #17571) colony was used to start an overnight culture in Luria-Bertani broth. 100 µl of this culture was used to inoculate the flow system.

At each experimental time point, flow was temporarily stopped and individual capsules were removed from the system. Coupons were removed from the capsules using sterile forceps, gently rinsed in 3 ml 0.1M HEPES (pH 7.3), and immediately processed for cell counting or scanning electron microscopy (SEM). For all types of analysis, the underside of the coupon was assayed so that analysis was performed on adhered biofilm cells, rather than cells that had settled from the medium.

3.3 Quantitation of microbial growth on the coupons and in the medium

Biofilm growth on the coupons was quantitated as described previously [17-19]. Briefly, coupons were scraped repeatedly into 45 ml of 0.5X M9 salts to remove the adhered biofilm, and then washed with 5 ml of 0.5X M9 salts. The solution was then homogenized for 30s to disaggregate the bacteria. The cell suspension was serially diluted and plated onto LB-agar by the drop plate method [18]. After incubating at 30°C overnight, colonies were counted and colony forming units (CFUs) were calculated per unit area. Two coupons were sampled per time point. Bacteria levels within the media were also quantitated by the drop plate method. Each experiment was performed three times; representative data is presented.

To calculate the rate of decline of biofilm CFU/cm^2 over time, a linear regression analysis was performed on time points between 7d and 28d for each of 3 separate experiments. Rates were averaged and the standard error was calculated. To calculate the percent of decline of biofilm CFU/cm^2 over time, we used the following formula: (CFU/cm^2 at 7d)/(CFU/cm^2 at 35d) *100. Percentages were calculated for each experiment and averaged; standard error was calculated.

3.4 Scanning Electron Microscopy (SEM)

Coupons were prepared for SEM microscopy essentially as described in Priester et al. [20]. Coupons were pre-fixed in 0.075% ruthenium red, 2.5% glutaraldehyde, 50mM L-lysine in 0.1M HEPES, pH 7.3; fixed in 0.075% ruthenium red, 2.5% glutaraldehyde in 0.1M HEPES, pH 7.3; washed three times in 0.1M HEPES, pH 7.3; post-fixed in 2% osmium tetroxide; and rinsed three more times in 0.1M HEPES, pH 7.3. Following staining and fixation, samples were taken through ethanol dehydration and dried through critical point drying (Tsousimis, Inc.) or air drying. Coupons were mounted onto SEM stubs using copper tape. Neat resin samples were coated with 50 angstroms of gold using a Southbay Technology coater where the samples were rotated and tilted during coating. Imaging was performed on an FEI Quanta 600 with a Field Emission Gun. Voltages for the imaging were at 15kV.

For SEM microscopy of cells agglomerated to carbon black, *P. fluorescens* was grown in 50 ml of 0.5X M9 with 10mM pyruvate and 0.01 g carbon black powder (Vulcan XC72; Cabot, Inc.) for 24h in a shaking incubator at 28° C. Samples were removed, centrifuged briefly, then the cell/carbon black pellet was taken through the same fixation, staining, and dehydration treatments as the coupons. After the final rinse, particles were dropped onto a SEM stub coated with carbon sticky tape, air dried, and imaged as above.

4. Results and Discussion

4.1 Impact of medium on biofilm growth and persistence on coupons.

A previous report by Kay et al. [16] examined the impact of medium composition on biodegradation of PU coupons. It was found that richer medium supported more microbial growth in the planktonic phase and resulted in greater substrate degradation. However, no quantitation of biofilm growth or persistence on the coupons was performed. Therefore, we examined the growth and persistence of *P. fluorescens* on an Air Force PU coating as a function of medium conditions. We first tested the ability of *P. fluorescens* to grow on PU coupons in the presence of M9 minimal salts, which contain phosphate buffered saline and ammonium chloride, but no carbon source. The only available carbon source was the coating on the coupon. Under these conditions, *P. fluorescens* grew rapidly, reaching maximum density by 4d (Fig. 2). The biofilm persisted for 35d, although the number of cells on the coupon, as well as in the medium, declined slowly over that time period. Due to the absence of any other carbon sources other than the coupon, this result suggests that *P. fluorescens* can potentially use the paint as a sole carbon source. However, the decline in biofilm cell density over 35d also suggests either that the paint may not be a preferred nutrient source or that other parameters limit the persistence of cells on the coupon surface. Such parameters could include increasing concentrations of toxic metabolic products.

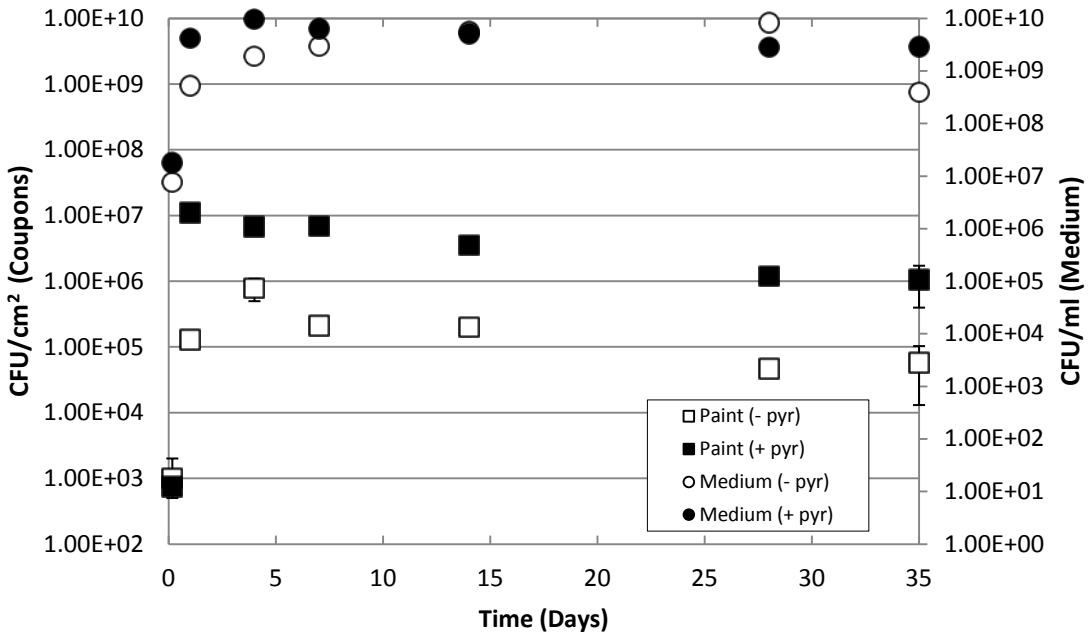

Figure 2. Growth of *P. fluorescens* on PU Coupons and Within Medium, With and Without Pyruvate. Growth of *P. fluorescens* on PU coupons (Paint) and within the medium (Medium) in which the coupons were incubated. Medium was M9 salts without (- pyr) and with (+ pyr) 10 mM pyruvate. Each point represents the average of 2 coupons. Error bars are standard error of the mean. This graph is representative of 3 independent experiments.

When pyruvate was added to the medium as a carbon source, growth on the coupons increased approximately 10-fold (Fig. 2). The number of biofilm cells on the coupon peaked sooner in the presence of pyruvate than in its absence, within 1d of inoculation. While these conditions resulted in an increase in the number of cells that persisted on the coupon over 35d, the addition of pyruvate at the beginning of the experiment did not support long-term persistence. The rate at which biofilm CFUs declined per day was much faster in the presence of pyruvate (239,719 CFU/cm^2 $\pm$ 29,341) than it its absence (40,552 CFU/cm^2 $\pm$ 29,341) (Table 1). However, biofilms grown in the presence and the absence of pyruvate lost the same proportion of cells over the course of the experiment, approximately 19% per day (Table 1).

Table 1. Comparison of Rates and Percent of of Biofilm Decline, With and Without Pyruvate

Treatment	Rate of decline		Percent of decline	
	CFUcm^{-2}/d	Std Error	%/d	Std Error
M9 - pyruvate	40,552	29,341	18.5	4.2
M9 + pyruvate	239,719	17,100	19.4	4.7

At most time points, the difference in CFU on the coupons was greater than the difference in CFU in the medium, a difference that is substantial considering that growth on the coupons was calculated based on area, while the medium was calculated per volume. These results demonstrate that the addition of a carbon source to the medium specifically increased the density of biofilm cells on the coupon. Therefore, carbon is a limiting factor in growth of biofilms on PU, even though PU is a potential carbon source [13, 14].

4.2 Impact of substrate on biofilm growth and persistence on coupons.

AS-P108 is a PU-based antistatic paint that is conductive due to the presence of carbon black particles. Previous research has evaluated the growth of biofilm on similar anti-static paint but did not evaluate whether the carbon black particles supported biofilm growth on the paint [14]. We have observed that *P. fluorescens* readily agglomerates to free carbon black particles present in the medium (Fig. 3); these particles could be a potential carbon source for the bacterium, particularly in nutrient poor conditions.

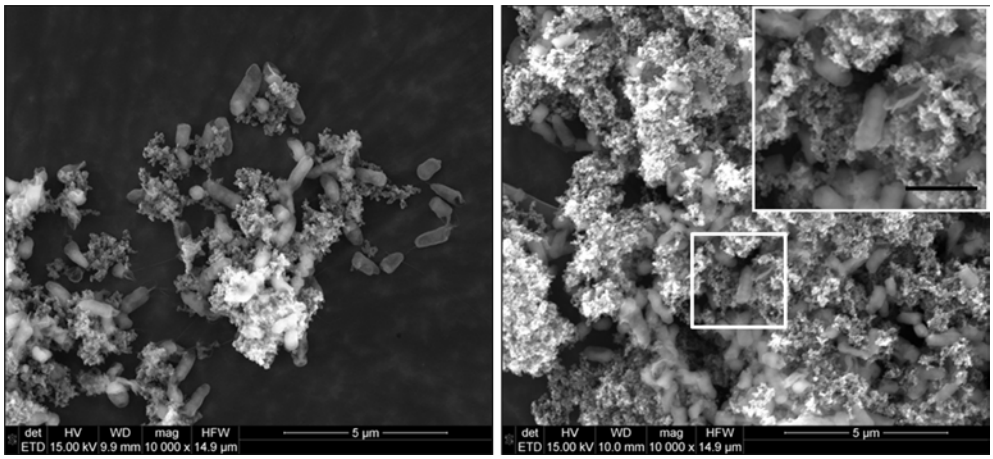

Figure 3. SEM Images of *P. fluorescens* Agglomerated to Free Carbon Black. Right and Left panels, scale bar = 5 μm. Right panel, inset is magnified view of white boxed area. Inset scale bar = 1 μm.

To evaluate the contribution of carbon black to biofilm growth and persistence, we created a set of coupons that were coated with neat resin, i.e., AS-P108 without the carbon black particles. When incubated in our flow system, we found no substantial difference in the biofilm growth or persistence on the coupons over 35d (Fig. 4). The result was the same whether or not the medium contained pyruvate as a carbon source (data not shown). This result suggests that PU, not the carbon black, is the main carbon source for the *P. fluorescens* in AS-P108.

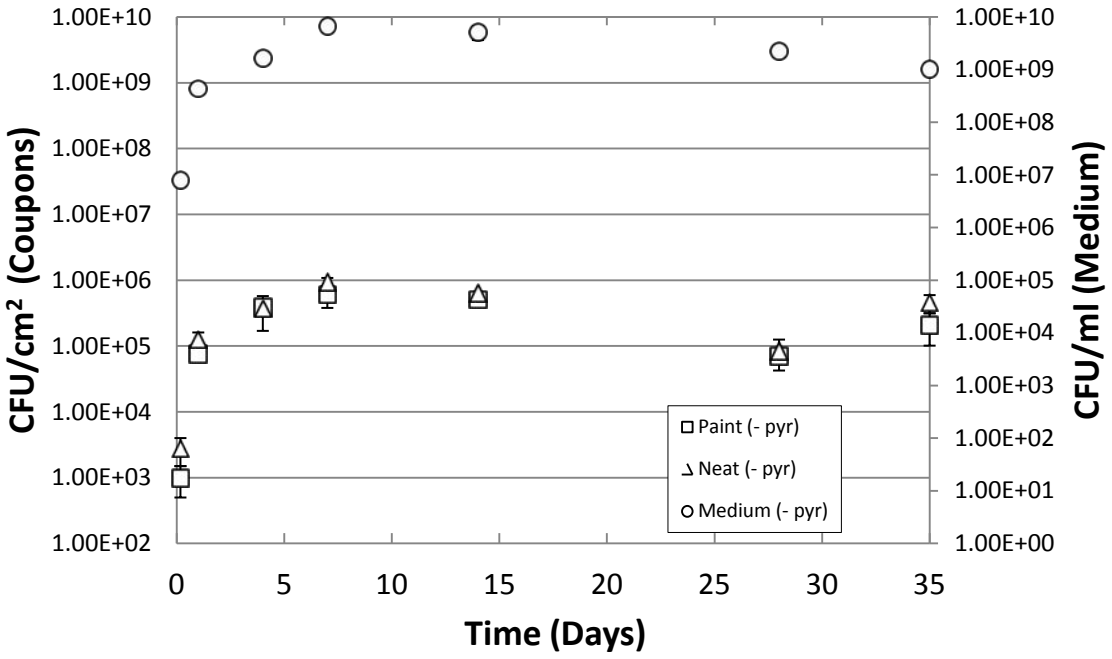

Figure 4. Growth of *P. fluorescens* on PU coupons and Within Medium, Paint vs. Neat Resin. Coupons consisted of AS-P108 paint containing carbon black particles (Paint) and neat resin (Neat), which is AS-P108 without the carbon black pigment. Medium was M9 salts without pyruvate (- pyr). Each point represents the average of 2 coupons. Error bars are standard error of the mean. This graph is representative of 3 experiments.

4.3 Evaluation of biodegradation of PU

At each experimental time point, the coupons were evaluated for PU degradation. We observed that after 35d of exposure, the PU had swelled significantly and was no longer firmly adhered to the coupon. This change in adhesion occurred on both paint and neat resin samples, regardless of whether the coupon had been exposed to sterile medium or to *P. fluorescens*; thus far changes to mechanical properties in the paint have not been quantitated. We also examined each coupon by SEM microscopy (Fig. 5). At early timepoints, *P. fluorescens* biofilms were monolayers. Biofilms became more three dimensional over the course of the experiment. EPS production and cell numbers were highest at 7d (data not shown), consistent with our quantitation results. Occasionally, cracking or pitting was observed near cells, but this damage was not consistent among experiments, and could not be separated from artifactual damage that may have been caused by fixation or drying processes required for SEM. There was no evidence of gross morphological damage, regardless of medium, substrate, or exposure length. It is important to note that we independently confirmed that the *P. fluorescens* strain used in this experiment was able utilize colloidal PU as a carbon source (data not shown). It is possible that damage, particularly pitting, may be obscured by the presence of the biofilm on the substrate, or that we have yet to identify the exact parameters that facilitate degradation.

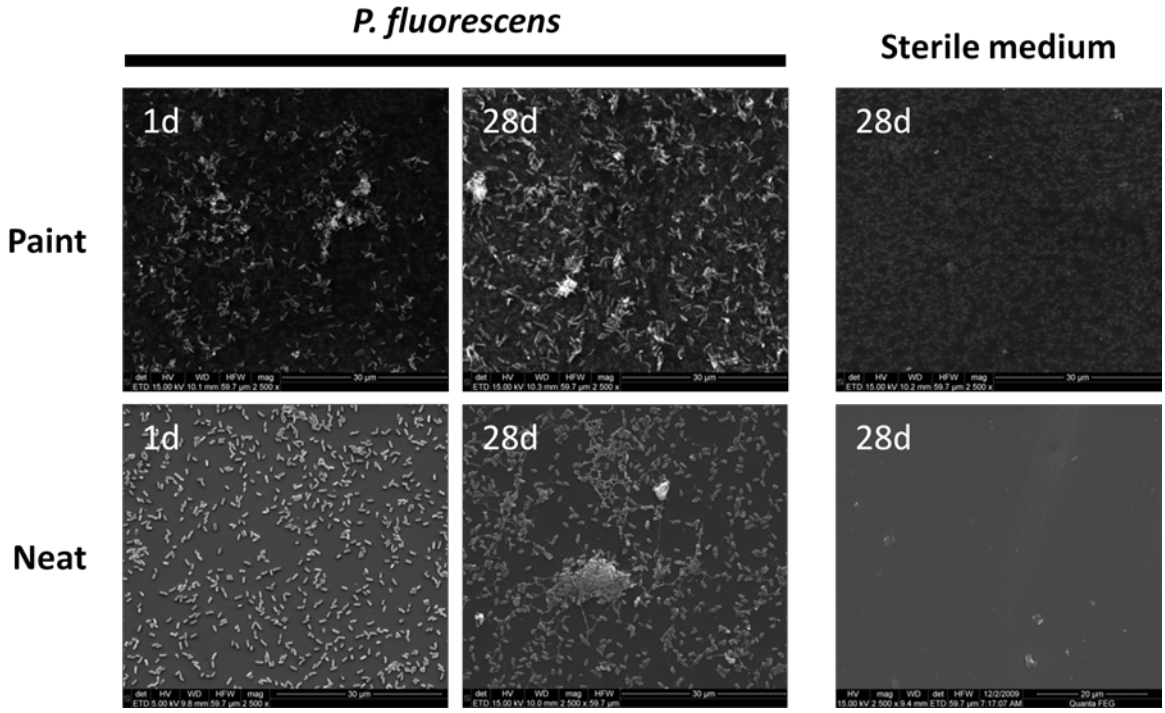

Figure 5. SEM microscopy of Coupons Exposed to *P. fluorescens* and Sterile Medium. SEM microscopy of coupons coated with AS-P108 (Paint) and neat resin (Neat). Coupons were exposed to *P. fluorescens* or Sterile medium (M9 salts + pyruvate) for 1d or 28d.

5. Conclusions

Air Force fuel systems are under constant attack by environmental microbes that degrade the fuel, the tanks, and polyurethane tank coatings. The purpose of this study was to evaluate the parameters that affect biofilm growth on Air Force polyurethane (PU) coatings so as to understand how these factors contribute to PU degradation. We chose to expose the coupons to cultures of *Pseudomonas fluorescens* for the following reasons: 1) Pseudomonads are biofilm-forming, facultative aerobes that are routinely found in aviation fuel systems; 2) *P. fluorescens*, as well as other Pseudomonads, have been shown to use PU as sole carbon source.

We found that *P. fluorescens* biofilms grew rapidly on PU coupons in the absence of any carbon source other than the coupon itself. However, biofilm growth increased when a small amount of pyruvate, a simple carbon source, was added to the medium in which the coupons were exposed. In both the presence and absence of pyruvate, the biofilm declined steadily over the course of the 35 day experiment; nearly 1 in 5 cells died on the coupon each day. This result suggests two scenarios, both of which may be true. First, although *P. fluorescens* can utilize the PU as a carbon source, it is not a preferred substrate. To metabolize PU, Pseudomonads must secrete enzymes that degrade high molecular weight PU polymers into smaller subunits. This is an energetically expensive process and is obviously less favorable than importing less complex amino acids or polysaccharides through membrane channels. It also implies that the cells must be metabolically quite active, which they are unlikely to be if they are starved or stressed significantly. Second, other parameters may have affected the persistence of the biofilm on the coupons. Such parameters could include the buildup of toxic metabolic products and release of toxic chemicals from the paint. In the case of exposures that occurred in the presence of pyruvate, it is likely that pyruvate concentrations declined over the course of the experiment, negating any initial beneficial effect it might have had on biofilm growth. To address these issues, future flow systems can either be refreshed with fresh medium periodically, or the flow system can be changed to a flow-through system, which would have a constant influx of fresh medium and no accumulation of metabolic waste.

In this study, we also evaluated the impact of the substrate on biofilm growth. The paint chosen for these studies is an antistatic paint that contains carbon black particles. These particles could be used a carbon source for the biofilm. We found that biofilms grew equally well on neat resin samples, which lacked the carbon black pigment, as they did on the carbon black-containing paint. This result suggested that the biofilm is most likely utilizing the PU, and not the carbon black, as a nutrient source.

We also evaluated the biodegradative effects of *P. fluorescens* on the coupons. Regardless of the conditions we tested, we could not find evidence of degradation of the coatings. No widespread damage was observed that was reproducible among experiments. Occasionally we observed pitting and cracking, but could not distinguish between artifactual damage due to SEM sample processing methods, and actual biodegradation. We routinely observed that the coating swelled over the course of the experiment, so that the paint was very poorly adhered to the coupon by day 35. This effect was observed upon exposure to *P. fluorescens* as well as to sterile medium. In future experiments, we will quantitate changes in mechanical properties of the coating following these exposures, to determine if there *P. fluorescens* causes any specific effects on the coating.

Our findings support the need to further characterize the factors that contribute to biodegradation. Growth of a biofilm on a coating is not an immediate predictor for the

degradative capacity of a biofilm. Biodegradation is caused by an interplay between biofilm cells, substrates, and the medium in which the biofilms are grown.

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